EDIBLE BARRIER

FIELD OF THE INVENTION

The invention relates to a barrier. More in particular the
invention relates to an edible moisture or flavour barrier
suitable for use in food products, comprising a cross-linked
biopolymer and a lipid material.

BACKGROUND OF THE INVENTION

Migration of moisture and flavour in food products forms a
serious problem because it negatively affects the appearance,
taste, freshness, shelf life and consumer satisfaction. In
order to prevent such migration, edible barrier materials have
been proposed. Currently available edible moisture barrier
technology is not suitable for effectively stopping moisture
migration in composite food products during shelf life. Lipid
material based moisture barriers lack physical strength and
flexibility and cannot withstand elevated temperatures during
processing. Hydrocolloid-based edible films potentially have
better tensile strength, but are not very effective because of
their hydrophilic nature. Upon drying, hydrocolloid films tend
to become rather brittle and hence lose their superior physical
properties. Combinations of hydrocolloid and lipid films have
been applied in alternating layers (laminate) to take advantage
of both systems, but require complex and expensive processing.

Thus, there is still a need for alternative or improved edible
barrier suitable for use in food products.

It is therefore an object of the invention to provide an edible
barrier suitable for use in food products, which does not have
one or more of the above mentioned drawbacks.

It was now surprisingly found that the above object of the
invention may be achieved by the edible barrier of the
invention, which is suitable for use in food products, and
comprises a cross-linked biopolymer and a lipid material, said

5 edible barrier having a thickness of about 2 to 1,500
micrometer. Preferably, the biopolymer is a cross-linked
pectin, chitosan or starch.

Moreover, the temperature stability of the barrier was found to

10 be excellent, they are stable from -20°C tot 150°C, freeze thaw
stable and cooking and frying stable.

WO02/071870 (Unilever) discloses a foamed product wherein
cross-linked pectin is homogeneously incorporated, as in food

15 product such as mousse or an ice cream.

The earlier, not pre-published WO04/000041 (Unilever) discloses
a method of preparing stable oil in water emulsions wherein a
ferulyolated compound is at least partly oxidized during or

20 after formation of the oil in water emulsion.

DEFINITION OF THE INVENTION
According to a first aspect of the invention, there is provided
an edible barrier suitable for use in food products, comprising

25 a cross-linked biopolymer and a lipid material, said edible
barrier having a thickness of about 2 to 1,500 micrometer.

According to a second aspect of the invention, there is
provided a composite food product comprising parts having

30 different water activities (aw), separated by the barrier
material according to the invention.

DETAILED DESCRIPTION OF THE INVENTION

The edible barrier according to the present invention comprises a cross-linked biopolymer and a lipid material, said edible
5 barrier having a thickness of about 2 to 1,500 micrometer. The barrier forms a film that can be used to prevent migration of moisture or flavour from one part of a food product to another part. It has been found that films comprising such cross-linked biopolymers have a high physical strength. If the biopolymers
10 are hydrocolloids, the films have surprisingly good adhering properties to a wide range of food matrices. The hydrocolloid films form effective aroma barriers. Barrier films with a high lipid content are very effective in inhibiting moisture migration. The lipid binding capacity of the film can be
15 further enhanced by hydrophobic modification, i.e. by attaching hydrophobic groups to the cross-linked polymers.

It was found to be especially attractive to apply enzymatic cross-link technology to stop water and/or flavour migration
20 from ingredients. In this technique, ferulyolated pectin or ferulyolated biopolymers or vanillin attached biopolymers such as chitosan-vanillin are covalently cross-linked.

It was found to be possible to enhance the physical strength of
25 the edible barrier film via cross-linking of the hydrocolloid polymers and to exploit this increased strength in combination with a high lipid binding capacity of the film due to strong interactions between the hydrocolloid network and lipids. Moreover, the chemical properties of the hydrocolloids allow
30 strong adherence to a wide range of ingredient surfaces.

Certain biopolymers containing ferulic acid groups attached to
their backbone are known to be gellable by oxidation. An
example of these polymers is pectin from a limited number of
plant sources. The gelling may be achieved by addition of an
5 appropriate amount of an enzyme of the oxidase type, e.g.
laccase or peroxidase. The ingredients of the application may
contain these enzymes which allows the process to occur without
addition of exogeneous enzymes. Moisture barriers can play an
important role to enhance product quality during storage of
10 composite food products. Thermodynamic instability of small
molecules in different compartments of the composite food
product drives migration into other compartments. The resulting
migration of moisture, flavour and colour causes deterioration
of the sensory properties of the different parts of the
15 composite food product. Application of the new barrier
technology at the surface of ingredients inhibits water,
flavour and colour migration resulting in a better consistency
of the ingredients and hence overall quality of the product.

Table 1

	film forming properties	physical strength	lipid binding capacity	moisture barrier properties	aroma barrier properties
Hydrocolloids	++	−	−	−	+
Cross-linked hydrocolloids	+++	+++	+	−	++
Entangled lipids	++	++		+++	−
Covalently linked lipids	++	++	+++	+	−
Covalently linked + entangled lipids	++	++	+++	++++	−

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The invention regards the composition and preparation of an
edible moisture barrier material. Such barrier consists of a
network of at least (covalently) coupled hydrocolloids.
Covalent coupling can be accomplished by means of cross-linking
5   agents like epichlorohydrin. Alternatively, covalent coupling
can be achieved via an oxidation reaction of polyphenolic
groups (e.g. ferulic acid residues) which leads to gel
formation or at least increased viscosity of the aqueous phase.
The gel forming capacity of e.g. pectins is for example
10 described in WO-A-98/22513 and WO-A-00/40098 and WO-A-96/03440.

Ferulic acid groups (4-hydroxy-3-methoxy-cinnamyl – groups) are
known to be capable of cross-linking in the presence of certain
oxidants (e.g. Oosterveld et al; oxidative cross-linking of
15 pectic polysaccharides from sugar beet pulp, Carbohydrate
Research 328; 199-207, 2000). The 4-hydroxyl-3-methoxy-benzene
part is the functional moiety of ferulic acid that is involved
in the oxidative cross-linking reaction. And hence cross-links
can be established between molecules with this type of (ortho-
20 methoxy-) phenolic group. In the oxidation process a new
covalent bond is formed between two individual ferulic or other
phenolic groups.

The term oxidant is used to indicate an oxidising agent, which
25 can be either a chemical oxidising agent or an enzyme. An
enzyme can be used alone or in combination with a co-oxidant
such as hydrogen peroxide.

The compound comprising ferulyolated (or similar ortho-mentoxy-
30 phenolic) groups is preferably a polymer, more preferably a
polysaccharide. Examples of suitable polymers include pectin,
chitosan, arabinan, galactan, cellulose derivatives,
galactomannans such as guar gum, locust bean gum, starches or

other polymers comprising hydroxyl groups which can be
esterified to a ferulic acid group. Alternatively ortho-
methoxy-phenolic can be covalently coupled to the carbohydrate
backbone. The polymers comprising ferulic acid groups can be

5   naturally occurring or synthesised polymers. Examples of
naturally occurring polymers with ferulic acid groups are sugar
beet pectin and arabinoxylanes isolated from cereals. Synthetic
processes to prepare polymers with ferulic acid groups
generally include esterification of ferulic acid to a free

10 hydroxyl group situated on the polymer backbone or on a sugar
substituent.

Alternatively, a phenolic group can be introduced in the
polymer chain via an imine bond between an amine group in the

15 polymer and an aldehyde function in the phenolic compound, as
in e.g. chitosan and vanillin.

In a highly preferred embodiment, the ferulyolated compound is
a pectin, even more preferred sugar beet pectin. The principal

20 building units of pectin are smooth homogalacturonic regions
and rhamnified hairy regions in which most neutral sugars are
located. Arabinose is the predominant neutral sugar. Galactose
is present in rhamnogalacturonan. 50-55% of the ferulic acid
groups are linked to arabinose units and about 45-50% of the

25 ferulic acid groups are linked to galactose residues.
Preferably, 15 to 80% of all ferulic acid groups are oxidised
in the final emulsion, after oxidation.

It is preferred that the majority of ferulic acid groups is not

30 oxidised before the oxidation during gel formation. Even more
preferred before gel formation at most 10% of all ferulic acid
groups are oxidised.

In another preferred embodiment the polymer is chitosan with covalently coupled vanillin moieties. The coupling of vanillin to the chitosan backbone can be achieved via a Schiff base.

In yet another embodiment, the polymer is cross-linked starch. It is also possible to use a cross-linked protein, or a cross-linked combination of one or more proteins and one or more carbohydrates.

The edible barrier according to the invention includes a lipid material and the oxidation of the ferulyolated polymers leads to network formation whereby the dispersed lipid phase is trapped as an o/w emulsion-layer. The lipid composition is dependent on the type of product and its processing conditions. The lipid material can be any edible oil fat or wax and is preferably selected from the group comprising sunflower oil, coconut oil, butter fat, rapeseed oil, olive oil, peanut oil or oils extracted from plant or flower material such as rose oil, and combinations thereof. Also fractionated oils and waxes are encompassed in the invention.

The oxidation may be accomplished by the action of a powerful chemical oxidant such as potassium periodate, potassium permanganate, or potassium ferricyanide.

Alternatively, the oxidation can be accomplished by use of an oxidising enzyme such as a peroxidase, a polyphenol oxidase e.g. catechol oxidase, tyrosinase, or a laccase. Peroxidases can be divided into those originating from plants, such as tomato peroxidase, fungi or bacteria and those originating from a mammalian source. Laccases are obtainable from a variety of microbial sources notably bacteria and fungi (including

filamentous fungi and yeasts), and suitable examples of
laccases include those obtainable from strains of Aspergillus,
Neurospora (e.g. N. crassa), Prodospora, Botrytis, Collybia,
Fomes, Lentinus, Pleurotus, Trametes (some species/strains of
5 which are known by various names and/or have previously been
classified differently), Polyporus, Rhizoctonia, Coprinus,
Psatyrella, Myceliophtora, Schytalidium, Phlebia or Coriolus.

Preferred enzymes are selected from the group consisting of
10 tomato peroxidase, horseradish peroxidase, soy bean peroxidase
and laccases that show a redox potential of preferably more
than 450 mV as described in E. Solomon et al, Chem Rev, 1996, p
2563-2605.

15 In case an enzymatic oxidising system is applied, the enzyme is
preferably added in the form of a solution or a dispersion in
an aqueous buffer system. The enzymes cited above are suitable
enzymes. Some enzymes, such as peroxidases require the presence
of a co-oxidant such as hydrogen peroxide for their activity.
20 The co-oxidant is preferably added separately from the enzyme
that requires its presence.

The amount of enzyme added is expressed in terms of activity
units. Preferably, the enzyme is present in excess. The amount
25 of enzyme added is preferably such that fast cross-linking
occurs. For a peroxidase the amount of enzyme added is
preferably from 10 to 100,000 units ABTS activity per ml of
liquid. In some food ingredients like e.g. fruits and
vegetables, the enzyme is endogeneously present and needs lower
30 or no external addition.

The oxidation is preferably carried out at a temperature of
from -20°C to 80°C, preferably 4°C to 70°C. It will be
appreciated that the best temperature depends on the oxidation
system that has been chosen.

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According to another embodiment the oxidising agent is added to
the aqueous phase which already comprises ferulyolated
compound, while the enzyme is endogenously present.

10 The amount of ferulyolated compound is preferably from 0.5 to 2
wt% (g ferulic acid per 100g pectin). The amount of
ferulyolated compound as a stock solution which is used for a
barrier is preferably from 6 to 10 wt% (g ferulyolated compound
per 100ml solvent). The solution can be sprayed or applied as
15 such at the surface of the ingredient / product. Alternatively
the ferulyolated compound is first applied and secondly a layer
of enzyme/ oxidising agent is added. Preferably, the layer is
dried in an oven or grill after the solution (barrier) is
applied and before the ingredients are used for the final
20 product. The barrier can also be applied as dry powder which is
a mixture of ferulyolated compound and oxidising agent(s).
Hydrogen peroxide can be added in solution or can be generated
in situ by means of glucose/ glucose oxidase addition.

25 Food products wherein the barrier may suitably be used are
preferably selected from the group consisting of leaking
(moisture or flavour or oil) ingredients such as vegetables
(tomato, salad), fruit, bread, fish and meat. The format of the
ingredient can range from native to pulp, dried gelled etc.
30

The barrier may further comprises optional ingredients such as
protein, salt, flavour, anti-microbials, components,

colourants, emulsifiers, acidifying agents, (co)-oxidants such
as hydrogen peroxide, and the like.
In order to achieve effective moisture barrier properties, the
barrier contains a lipid material, such as lipids entangled in
5   the matrix. The hydrophobicity of the barrier may also be
increased by attaching lipid molecules to the polymer backbone.
Attaching lipid moieties can be accomplished by covalent
linkage of glycerol moieties containing one or two fatty acid
chains and a ferulic acid, esterified to one of the glycerol
10 hydroxyl groups to the polymer backbone. Alternatively,
gossypol can be attached to the polymer backbone via oxidative
coupling. Fatty acids can be directly bound to the polymer
backbone by esterification (cf esterified starch).
Alternatively, the hydrophobic moiety can be covalently coupled
15 to the polysaccharide backbone by any covalent coupling
reaction known in the art (e.g. Schiff base coupling).

A higher lipid content of the film can be achieved by
incorporation of lipid material in the hydrophobically modified
20 polymer as described above.

The invention will be further illustrated in the following non-
limiting examples.

25 **Example 1**
Moisture barrier films
Materials:
Sugar beet pectin was obtained CP Kelco, GmbH, batch genu β
pectin. Chitosan and sodium caseinate were obtained from van
30 Erven BV. Soybean peroxidase and vanillin were obtained from
Quest. Peroxidase is a non-GM, food grade Biobake Wheat from
Quest, the Netherlands. The cofactor hydrogen peroxide used is
30% solution of Merck, Germany. Glucose oxidase, non-GM, food

grade from Amano in combination with glucose and Biobake wheat were used for some examples. Novozym 435 lipase and pectinex ultra SP-L were obtained from Novozymes. Beeswax, carnauba wax and ethylferulate were obtained from Sigma Aldrich Chemicals. Solid fats MGLA41, coco nut oil, PO58 flakes, and RPLE70 were obtained from W.T. Hogervorst, SCC, URDV. All other chemicals used were obtained from Merck.

Film components: Chitosan modified with vanillin (Chitosan-V)

Synthesis A
Vanillin was dissolved in 97% Ethanol and chitosan powder was added to this solution. The resulting suspension was incubated for 1 hour up to 7 days at 65°C, 200 rpm. The ratio chitosan: vanillin (w/w) varied in a range between 2:1 up to 500:1 and the amount of ethanol used, was adjusted to maintain a moving suspension. After the incubation, the suspension was filtered and the filtrate was washed with ethanol to remove free vanillin. Finally the filtrate was dried on air.

Synthesis B
Chitosan (0.8 g) was dissolved in 90 ml of 2% acetic acid-methanol (1:2, v/v). A solution of 2,75 g vanillin in 10 ml methanol was added and the mixture was incubated for 24 hours up to 7 days at room temperature to obtain a yellow hydrogel. The gel was dried and washed with water and methanol several times to remove free vanillin.

Analysis: The degree of vanillin substitution was calculated via the average molecular mass of a chitosan-monomer based on a total N-determination. The following formulas were used:

$$N(g/g) = M_N/M_{polymer}$$

N (g/g) is experimentally determined and M_N is known
→calculate $M_{polymer}$

$$M_{polymer} = (v * M_{chitosan\ monomer} + M_{vanillin})/v$$

$M_{vanillin}$ and unmodified $M_{chitosan\ monomer}$ are known → calculate v
(degree of vanilisation)

$$v = M_{vanillin}/(M_{polymer} - M_{chitosan\ monomer})$$

Besides an element analysis, modified chitosan was also
unsuccessfully analysed by MALDI-TOF MS. The chitosan molecules
were too large and molecular weights were too divers to obtain
reliable results.

Sodium caseinate modified with vanillin (Na-cas-V).
200g sodium-caseinate was added to a solution of 7g vanillin in
4 litre demineralised water, pH adjusted to 7.5. The solution
was cooled to -80°C and freeze dried for approximately 10 days
at 100 mbar. Yellow powder was obtained and subsequently
dissolved in 2l demineralised water. Then 2l ethanol was added
to the solution to precipitate the protein and separate it from
free vanillin. The resulting suspension was centrifuged for 20
minutes at 4,500 rpm. If the supernatant was still turbid after
centrifugation, the pH was further decreased to pH 4 and
centrifugation was repeated. After centrifugation the pellet
(modified protein) was cooled to -80°C and freeze-dried for
another 2 days to obtain dry product.

Feruoylated triglycerides (FG)
Synthesis: 40 g of sunflower seed oil and 3.5 g ethylferulate
were dried for approximately 16 hours on blue silica. After
this drying step, ethylferulate was dissolved in the dried

sunflower seed oil and 3.5 g lipase (Novozyme 435) was
suspended into this solution. The mixture was sealed and
incubated at 65°C, 200 rpm for 7 days. Subsequently the mixture
was separated on a silica gel 60 extra pure (Merck) column with
5 an eluens increasing in polarity. The first fractions were
eluted with petroleum ether: diethyl ether (v/v) 4:1 followed
by ratios 7:3, 3:2 and finally only diethylether was used. In
total 12 fractions of each approximately 200 ml were collected
and the eluens was evaporated to obtain concentrated fractions
10 for analysis.

Analysis:
TLC: 10 µl of each fraction was analysed on TLC. The samples
were pipetted on a silica glass plate and eluted with
15 toluene:diethylether (v/v) 4:1. The silica plate was dried on
air and components containing ferulic groups were identified by
UV fluorescence.

HPLC: Fractions were analysed on a C18 HPLC column with an
20 isocratic flow of 40/60 (v/v) acetone (containing 1% glacial
acetic acid) / acetonitrile. Samples were prepared by
dissolving 10 µl of each fraction in 1 ml acetone and remove
all solid particles by filtration over a silica gel 60 extra
pure column. As a control, HPLC spectra of ferulic acid,
25 ethylferulate, oleic acid and glycerol were recorded. The
presence of ferulic residues was monitored by UV-detection at
325nm and the presence of lipid components was monitored at
360nm. A reference (detection of components) was monitored at
450nm. All fractions that contained the feruoylated glycerides
30 were combined.

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LC-MS-MS: All measurements were carried out on the Quattro-II using HPLC-MS and MS-MS in positive electrospray mode. To support the ionisation, ammonium acetate was added post-column. More details are given in Appendix I.

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Activity assay (ABTS assay)

Add 100 µl 20 mM ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) stock solution made in indicated buffer) to 880 µl 25 mM phosphate buffer, pH 6.0. Incubate for 5' at 30°C. Add 10 µl 100 mM hydrogen peroxide. Start the reaction by addition of 10 µl enzyme (diluted in such a way that a linear curve could be measured). Measure the formation of ABTS radical at 414nm using a spectrophotometer. Specific activity is defined as: µmol ABTS oxidised per minute per mg protein at pH 6.0

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Polymer modified with feruoylated glycerides

Synthesis

The amount of feruoylated glycerides that was used for the coupling reaction to the polymer could be varied in different molar ratios. The following formulas were used for calculating these molar ratios:

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$$\text{Chitosan-V (g)} / M_{polymer} = \text{Chitosan-V (mol)}$$

25

$$\text{Chitosan-V (mol)} / v = \text{bound vanillin (mol)}$$

$$\text{Ratio FG:vanillin} * \text{bound vanillin (mol)} = \text{FG (mol)}$$

The desired amount of FG's was dissolved in ethanol and slowly added to the stirred polymer solution. Subsequently enzyme was added, also during mixing and finally peroxide to start the cross-linking reaction. Amounts of enzyme and peroxide are dependent on the nature and concentration of the polymer and are described in more detail in paragraph 3.2. When the

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modified polymer was used as an emulsifier, the final
percentage of pectin had to be lower than 1% and chitosan-V had
to be lower than 0.25% to prevent gelation of the solution.

5 Analysis
TLC analysis was used to monitor the coupling reaction. Before
and after addition of the peroxide, 15 µl sample was analysed
on a silica plate. Spots were eluted with toluene:diethylether
(v/v) 4:1 and the disappearance of free FG spots was used as an
10 indicator for a successful coupling reaction.

Fatty acid analysis was used to examine the coupling product of
the polymer and the modified triglycerides. After the coupling
reaction, 0.75 ml of the reaction product was separated from
15 any free FG left on TLC with eluens toluene:diethylether (v/v)
4:1. The lowest spot containing the modified polymer was
extracted from the silica in a acetic acid buffer pH 5.5 and
0.01 g lipase (Novozyme 435) was added. After incubation for 2
hours at 65°C, 900 rpm, the mixture was centrifuged. The
20 supernatant was concentrated by evaporation and a free fatty
acid analysis NEFA-C, ACS-ACOD method (Wako) was performed.

Functional test
Emulsifying properties of the modified polymer were used as a
25 functional test to check whether the FG was successfully
coupled to the polymer backbone. For this analysis 10µl FG was
dissolved in 1 ml EtOH and added to a 3ml chitosan-V solution
to finally obtain a 0.125% chitosan-V, 25% EtOH, 0.0625% acetic
acid solution. During mixing, 2.5 10µl pox was added and
30 finally 10µl peroxide to start the cross-linking reaction. The
mixture was incubated for 5 minutes at room temperature and
subsequently 1ml of the solution was added to 2ml sunflower
seed oil containing 1% b-carotene. The mixture was vortexed for

1 minute at maximum speed and the stabilisation of the
resulting emulsion was monitored during 48 hours. Emulsions
were analysed at various intervals with Light Microscopy (LM).

5 <u>Films</u>
Film synthesis
All films were based on a cross-linked gel that was cast onto a
Teflon coated glass plate, aluminium mould of 5*5 cm and 0.3-
2.5 mm depth, or a plastic weighing cup. These cast gels were
10 dried into an incubator at 50°C, an air oven or at room
temperature. Gel synthesis of three types of films, (cross-
linked, hydrophobic entangled and hydrophobic covalent
modification) will be described in the following three
paragraphs.
15

<u>Cross-linked films</u>
Pectin solutions were made by dissolving pectin in water (max
9% w/w) and adjusting the pH during stirring with 1 M NaOH to
pH 5.5. Chitosan-V was dissolved in 1% acetic acid (max 2% w/w)
20 and the pH was also adjusted with NaOH to pH 5-5.5. An amount
of soybean peroxidase (enzyme) was added to the polymer
solution during stirring and finally the peroxide was added to
start the cross-linking reaction.

25 <u>Hydrophobic entangled gels</u>
Hydrophobic entangled gels were synthesised by adding the
hydrophobic component to the polymer solution and turrax the
mixture for 1 minute to obtain a homogeneous emulsion. If a
solid fat or wax was used, the mixture was heated in a water
30 bath before turraxing, to melt the solid component. Solutions
containing protein, sterols, ethanol, glycerol (1% gel volume)
or emulsifiers (tween 80, span 80, lecithin 1% gel volume) were
also added to the polymer solution/emulsion during turraxing.

An overview of used hydrophobic components is shown in table
2.1. When all components were mixed, enzyme was added and
finally peroxide to start the cross-linking reaction. Denatured
protein films were obtained by heating the protein containing
gel at 95°C for 30 minutes to denature present proteins. Some
films contained digested chitosan-V polymer. For this purpose,
the polymer was incubated with pectinex, a pectinase enzyme,
for 2 hrs at 37°C.

Table 2.1 *Overview of various hydrophobic components in
hydrophobic entangled films*

Polymer	Liquid oil	Solid fat (fatty acid)	Wax	Protein solution	Sterol
Chitosan-V 10:5 0.5 – 2%	Sunflower seed oil Olive oil	MGLA41 Coco nut oil PO58 Flakes RPLE70 Oleic oil Lauric acid	Beeswax Carnauba wax	Na-caseinate –(V) egg protein	γ–oryzanol β–sito-sterol
Chitosan-V 10:1 0.5 – 2%	Sunflower seed oil	Oleic acid	Beeswax	–	γ–oryzanol β–sito-sterol
Pectin 2 – 4%	Sunflower seed oil Olive oil	Coco nut oil PO58 Flakes RPLE70 Oleic oil	Beeswax Carnauba wax	Na-caseinate –(V) egg protein	γ–oryzanol β–sito-sterol
Chitosan-V 10:5 Pectinex digested	Sunflower seed oil	Coco nut oil PO58 Flakes RPLE70	Beeswax	Na-caseinate	–

Covalently coupled gels

Covalently modified polymer gels were synthesised according to
the same protocols as hydrophobic entangled films, only the

solution/mixture added to the polymer during turrax contained a
component with a phenolic group that could be recognised by the
soybean peroxidase. In table 2.2, three components are listed
that were used in covalently modified films and various
5 combinations.

Table 2.2 *Overview of covalently coupled hydrophobic components
in films*

Compound	Solvent	In combination with
Na-caseinate-V	H_2O (pH 7.5)	Lipid, fat, Feruoylated Glycerides
γ-oryzanol	Ethanol	Lipid
Feruoylated glycerids	Ethanol	Lipid, fat, wax, Na-caseinate (V)

10

Film characterisation

After film synthesis, all intact, smooth films were analysed by
a water vapour permeability assay. Films that gave good results
15 were subsequently tested for their solubility and swelling
percentage and finally some films were analysed with light
microscopy. Protocols for these assays are described in the
following part.

20 **Water Vapour Permeability (WVP) assay**

The water vapour permeability was determined by gravimetrical
analysis. Cups with a diameter of 2 cm^2 were filled with 10 ml
demineralised water and sealed with a piece of film. The films
were completely sealed to the cup with parafilm or wax and were
25 weighted to determine initial weight. Cups were places in a
dessicator filled with dried blue silica to create a relative
humidity gradient of 0% in the dessicator up to 100% within the
cups. During 4-12 days, weight loss of the sealed cups was
determined and plotted against time. Slopes of these weight
30 loss curves (g/day) were compared to the slope of the unsealed

cup and this number was considered as relative water vapour permeability. Experiments showed that this slope did not changed significantly between day 2 to 5. After 5 days, the silica started to get saturated and was replaced by dry silica.

Thickness

Film thickness was measured with a digital thickness measurement (Mitutoyo) to the nearest 0.01mm at 10 random positions. An average of the 10 values was used to calculate film thickness.

Film swelling and solubility test

A piece of film sized 1 cm x 1 cm was cut and dried in an oven at 90 °C for 3 hours and weighted to obtain the initial dry weight. The piece of film was placed into a Falcon test tube, containing 10 ml demineralised water. The tube was incubated at 37 °C, 100 rpm for 24 hours. After incubation, the content of the test tube was filtered and total weight of the pre-weighted filter and film was determined to obtain a total swelling percentage according to the following formula:

$$Sw\% = [(\text{weight wet film} + \text{filter} - \text{weight wet filter})/\text{initial weight film}] \cdot 100\% - 100\%\%$$

Subsequently, the wet filter and film were dried in an air oven at 90°C for 3 hours and weighted again. Total film solubility was determined according to the following formula:

$$Sol\% = 100\% - [(\text{dry weight film+filter} - \text{dry weight filter})/\text{initial weight film}] \cdot 100\%$$

Microscopy

Films and emulsions were examined with light microscopy. Film
samples were prepared by cutting a piece of film sized 2.5 mm x
2.5 mm, placing it on an object glass and sealing the cover
glass with colourless nailpolish. For the emulsion samples, 0.1
ml of the stable emulsion was pipetted onto the object glass
and sealed with a cover glass. Both samples were examined with
enlargements of 10x, 20x and 40x.

Films

Film synthesis

Films were synthesised as described above with various
components in different combinations and concentration ranges.
In table 3.1 applied enzyme and peroxide concentrations are
mentioned that were used for the various polymer solutions.
Higher concentrations of peroxide were used for chitosan cross-
linking, because more covalent bonds could be formed between
the vanillin side chains (reaction mechanism 1.2.1). On the
contrary, enzyme concentrations were lower compared to pectin
cross-linking because of a higher rate of chitosan gel
formation. Besides amounts, the order of component addition
appeared to be very important. Various experiments had
indicated that it was crucial to add enzyme before peroxide
during chitosan cross-linking. If peroxide was added before the
enzyme, polymer gelation did not occur. In table 3.2 an
overview is given of concentration ranges of various film
components that were found to result in stable films.

Table 3.1 *Overview of polymer solutions and enzyme and peroxide concentrations in gels*

Polymer solution	Concentration stabile gel and dried film	10% Enzyme solution (μl/ml gel)	1 M H$_2$O$_2$ (μl/ml gel)
Chitosan-V (10:5)*	0.5%-2%	0.1	5-20
Chitosan-V (10:1)	0.5%-2%	0.1	5-20
Chitosan-V (10:0.2)	0.5%-2%	0.1	5-20
Pectin	2%-4%	1	0.5-8

* w/w ratio chitosan:vanillin during chitosan-V synthesis

Table 3.2
Overview of concentration ranges of various components to obtain stable films

Polymer	Liquid oil % gel volume	Fatty acid % gel volume	Solid fat % dr wt film	Wax % dr wt film	Protein solution % dr wt film	Ethanol % gel volume
Chitosan-V 10:5 0.5 - 2%	0 - 30%	0 - 30%	0 - 80%	0 - 80%	0 - 80%	0 - 67%
Chitosan-V 10:1 0.5 - 2%	0 - 5%	0 - 5%	0 - 80%			0 - 67%
Pectin 2 - 4%	0 - 5%	0 - 5%	0 - 80%	0 - 80%	0 - 80%	0 - 67%
Chitosan-V 10:5 Pectinex digested	0 - 5%	0 - 5%	0 - 80%	0 - 80%		0 - 67%

Various synthesised films were divided into films containing entangled components and films that were covalently modified.

Additives:

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To make the film more flexible, glycerol 400µl or 800µl
glycerol was added before turraxing. To promote formation of
the emulsion, emulsifiers were added to the reaction mixture
before turraxing. Various amounts of mono/di glycerides and
5   lecithin were used as emulsifiers. To decrease the water vapour
permeability of the films, additional hydrophobic compounds
were added to the chitosan-vanillin solution before turraxing.
For example films were made containing β-sitosterol and γ-
oryzanol. The last compound is a mixture of sterols esterified
10 to ferulic acid. These compounds could also be cross-linked by
the enzyme to the chitosan network and increase the
hydrophobicity and network complexity, thereby decreasing the
water vapour permeability. In addition, ferulic acid trans-
esterified to triglycerid (mono or di ferulic glycerides) can
15 also be covalently cross-linked to the polymer network to
increase the hydrophobicity and water vapour permeability.

Film properties
In the introduction, three ways were described to obtain a
20 water barrier film using cross-linking technology. The same
format will be used to describe results of water barrier
properties of various synthesised films. Hydrophobic components
were added and finally hydrophobic components were covalently
bound to the polymer backbone to increase hydrophobicity.
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Hydrophobic entanglement
Several films with hydrophobic entangled components like oil,
fats and waxes were synthesised and analysed in a water vapour
permeability assay and solubility test. Besides film thickness,
30 the concentration of hydrophobic component was also crucial for
the water vapour permeability properties of the tested films.
When the concentration hydrophobic entangled component was

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increased, the water barrier properties were increased significantly.

In table 4 an overview is given of some water vapour
5   permeability assays in which pectin and chitosan films with or
without hydrophobic entangled components were tested. In the
table a the large difference between normal polymer films and
films containing entangled oil is shown. A water vapour
permeability value of almost zero was reached with pectin films
10 containing oleic acid, having a thickness around 1 mm. The same
trend was observed for films containing entangled wax and/or
solid fat. It is also clear that the wax/fat and oil containing
films obtained very similar barrier properties.

15   **Table 4**
                      Overview WVP analysis

NB.   – Percentages are v/v gel volume

      – Ratio's are w/w dry weight film

20   – Films are based on 20 ml 2% polymer gel

      – Unless mentioned different addition FG: 0.5 ml

Film Composition	Thickness	relative water vapour permeability
Pectin	0.060	1.0000
chitosan-vanillin	0.060	1.0000
pectin, 10% SO	0.915	0.1154
pectin, 20% SO	1.313	0.0385
pectin, 10% SO	0.419	0.2642
pectin, 10% SO	0.915	0.2264
pectin, 7.5% SO	0.434	0.2639
pectin, 2.5% SO	0.437	0.2500
Chit-V 10% SO	0.612	0.2639
Chit-V	0.696	0.1944
Chit-V 30% SO	0.324	0.2778
Chit-V 20% SO + 0.12 emulsifier	0.313	0.2786
pectin 1% SO + oryzanol 16:1	0.207	0.2556

Chit-V 20% SO + 20% EtOH	0.337	0.2556
Chit-V + 20% SO + beta-sitosterol 16:1 dry wt.	0.337	0.2778
Chit-V + 20% SO + beta-sitosterol 16:1 dry wt	0.444	0.2778
pectin + carnauba dry wt 1:2	0.437	0.2444
Chit-V 1% SO + beeswax dry wt 2:1	0.311	0.2360
chit-V 1% SO + 20% EtOH	0.177	0.2809
Chit-V 1% SO + oryzanol 16:1 dry wt	0.178	0.2697
Chit-V 1% SO + oryzanol 10.6:1 dry wt	0.202	0.2584
Chit-V 1% SO + 2.5 ml 3% NA-cas-V	0.215	0.2809
Chit-V 1% SO + 1 ml 3% NA-cas-V	0.19	0.2921
Chit-V 1% SO + 5 ml 3% NA-cas-V	0.232	0.2809
Chit-V 1% SO + 2.5 ml 3% NA-cas-V	0.282	0.2921
pectin + 9% SO + 1 ml 3% Na-cas-V + 20% EtOH	0.207	0.2035
pectin+ 10% OO + 20% EtOH	0.15	0.2124
pectin+ 10% SO + 20% EtOH	0.217	0.1947
pectin+ 10% SO	0.205	0.1858
Chit-V + 20% SO	0.424	0.2475
Chit-V + 20% SO + FG	0.464	0.2574
Chit-V+0.4 g coco (EtOH) +10% SO	0.236	0.2482
Chit-V+0.4 gr BW+ 2.5 ml EtOH+10%SO	0.321	0.2774
Chit-V+0.4gr PO50+ETOH+10%SO	0.297	0.2628
Chit-V+ 1 g BW + tween	0.177	0.2828
Chit-V + 2.5 g BW + FG	0.4	0.2727
Pectin + 10% oleic acid	1.457	0.0149
Pectin + 7.5% oleic acid	0.948	0.0373
Chit-V + 1.25 g BW + 1.25g PO58 + FG (1ml)	0.36	0.2462
Chit-V + 2.5g BW + FG (1 ml)	0.528	0.2462
Chit-V + 2.5g coco + FG (1.5 ml)	0.416	0.1791

Results of solubility analyses.

A large difference was observed between chitosan and pectin films. Pectin films showed high solubility compared to chitosan films. When hydrophobic components were added, a decrease was observed in film solubility. This effect was the same for both polymer films, only chitosan films reached significantly lower solubility values. Addition of protein had the opposite effect;

in general film solubility was increased when protein
concentration increased.

Example 2

5 Cheese - Yeast extract
   A new savoury spread, consisting of cheese and yeast extract or
   vegetable protein hydrolysate, with an original two coloured
   appearance was developed. Over time, however, colour migration
   caused serious appearance problem. Water migration occurred
10 from the high water containing cheese (aw =+/- 0.8) to the low
   water containing yeast extract (aw =0.2-0.4). Besides water
   migration, also the dark brown yeast extract colour migrates
   into the cheese phase, leading to one large brown coloured
   transition layer between the two components. An edible layer
15 was used to prevent dye and water migration. Chitosan films
   containing up to 50% lipid appeared to be very successful.
   Based on these results, it can be concluded that chitosan with
   a high lipid content can prevent water migration between two
   components with different water activities.
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   The Method for preparation of Chitosan-Vanillin films:
   Chitosan-vanillin film containing 20% (v/v) sunflower seed oil:
   5 ml sunflower seed oil was added to 20 ml 2% Chitosan-vanillin
   solution in 1% acetic acid which was adjusted to pH 4.5 with
25 NaOH. The solution was emulsified with an ultra-turrax T25
   (9,500 min^{-1}) for 5 minutes and during mixing add 2 µl enzyme
   (soy peroxidase). When the enzyme was homogeneously distributed
   20 µl 1M H_2O_2 was added. The solution was immediately cast on a
   Teflon-coated glass plate. The film is dried at 50°C and peeled
30 of from the glass plate.

   The lipid content can vary from 0% up to 50%.

Instead of sunflower seed oil, films were also synthesised
containing various amounts of olive oil and oleic acid.

Example 3

5 **Salami sausages**

Due to its adhering properties it is not possible to form a
homogeneous layer covering the complete sausage surface with
non-cross-linked pectin. The use of cross-linked pectin,
however, resulted in a film with amazingly good surface

10 adherence properties, probably due to covalent interaction
between pectin molecules and tyrosine groups of proteins
located on the sausage surface.

The pectin layer was applied on the sausage by dipping it into

15 a 4% pectin solution containing enzyme (1ml/100ml) and 25%
ethanol. Ethanol will decrease drying time and at the same time
increase solution viscosity, which promotes the adherence of
the gel onto the sausage. Subsequently the dipped sausage was
sprayed with a solution containing 1 mM peroxide and 2% pectin

20 (maximum concentration for spraying device) and dried in a hot
air oven for 4 hrs at 40°C. The appearance of the pectin coated
sausage is the same as the uncoated one.

The coated and uncoated sausages were tested on their

25 lipid/water release at room temperature and after 10 minutes
incubation at 100°C. The uncoated sausages lost oil which was
visible on a supporting paper tissue, whereas the coated
sausage did not lose any oil under the same conditions.

30 **Example 4**

Edible coating on biscuits

Biscuits were coated with cross-linked pectin-films and cross-
linked chitosan films containing lauric acid. Samples were
placed in a dessicator at RT containing a saturated NaCl
solution (80% RH). The weight increase due to water adsorption

5 was measured as a function of time. The results are shown in
Figure 1. During the first 6 days, the weight increase of
chitosan-coated biscuit (squares) was significantly lower than
the weight increase of the pectin coated biscuit (triangles).

10 **Example 5**
   Films of vanillin coupled Chitosan mixed with lipid
   A chitosan-vanillin (ChitV) solution was prepared as described
   earlier (synthesis B). The concentration of ChitV in the
   produced ChitV-solution was 3%. Synthesis of ferulyolated tri-

15 and diglycerides (FG) was described earlier.

   Film Synthesis:
   A homogeneous emulsion was prepared by mixing the ChitV
   solution with sunflower oil and/or wax (Cotebar A from Loders

20 Croklaan was used), and in some cases with ferulyolated tri-
   and diglycerides (FG) or Hymono 8903 (highly saturated
   monoglycerides from Quest). Mixing was done at high speed using
   an Ultra-Turrax for 1-5 minutes.

   If waxes were used, they were melted first and the other

25 components and the equipment used were warmed up in an oven
   before they could come in contact with the molten wax. Then
   enzyme (soybean peroxidase, Biobake from Quest) is added and
   after homogeneous distribution the cross-linking reaction is
   started with the addition of 1M hydrogen peroxide stock

30 solution. For a 6g-film 15µl (~2.5mM) were used. To see if the
   amount of hydrogen peroxide in the film is high enough for
   gelling, an indicator paper was used. The amount of enzyme and

H$_2$O$_2$ varied with the amount of starting material. Thin film layers on gelbond® film (from FMC Bioproduct, USA) which is a agarose coated polyester, which were dried on air or in the oven for several hours between 65°C and 100°C (conditions are

5   shown in Table 5). After drying the thickness of the films was measured and the water vapour permeability was measured. Results of the permeability (P) and the relative water vapour permeability (WVP) of different Chitosan-vanillin/lipid films are given in Table 5. The different compositions of the films

10   are given in %wt/wt of wet films (composition before drying).

Table 5

Film no./drying conditions	% 3%Chitosan	% SF	% Cotebar A	% FG	Hy-mono %	d [mm]	P [e^{-11} g/ (s*m*Pa)]	Rel. WVP [%]
1.	59.2	19.7	19.7	1.3	0	0.25	6.64	5.19
2.	59.2	19.7	19.7	1.3	0	0.62	7.61	4.80
3./4h 100°C	59.2	19.7	19.7	1.3	0	0.36	3.49	1.55
4./4h 100°C	59.2	19.7	19.7	1.3	0	0.09	2.00	3.55
5./4h 100°C	59.2	19.7	19.7	1.3	0	0.55	7.29	2.11
6.	42.3	35.5	1.1	21.1	0	0.43	8.72	5.01
7./4h 100°C	59.2	39.5	1.3	0	0	0.54	7.83	3.58
8./24h 65°C	60	20	20	0	0	0.37	9.13	4.2
9./	59.7	19.9	19.9	0	0.5	0.50	7.65	2.01
10.	59.7	19.9	19.9	0	0.5	0.06	6.08	n.d.
11	60	40	0	0	0	0.18	6.19	n.d.
12./25% EtOH	65	10	0	0	0	0.06	6.48	n.d.

n.d. is not determined

The water vapour permeability measurements show that the
chitosan-vanillin/lipid films had very good P-values thus a
very low permeability.